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Structural basis of Na⁺-independent and cooperative substrate/product antiport in CaiT

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1. Background

L-carnitine is an essential metabolite in animals, plants, and prokaryotes. *Escherichia coli* and related bacteria, such as *Proteus mirabilis*, use L-carnitine (R(-)-3-hydroxy-4-trimethylaminobutyrate) as an electron acceptor under anaerobic growth conditions and convert it to γ -butyrobetaine¹⁻⁴. Mammalian cells have similar organic cation/carnitine (OCTN) transporters (fig. S1). *E. coli* CaiT is a constitutively active, highly specific L-carnitine/ γ -butyrobetaine antiporter, which works in both directions⁵. CaiT belongs to the BCCT (betaine, carnitine, choline transporter) family, which all transport substrates that contain a quaternary ammonium group.

1.1 Trimer architecture

PmCaiT and EcCaiT both form compact trimers of three identical protomers (fig. S3A). A hydrophobic cavity on the cytoplasmic side contains ordered detergent (fig. S3B) and would be filled with – most likely also ordered – lipid in the membrane. As in BetP, the long, curved α -helix 7 (H7) runs along the periplasmic membrane surface. This helix and the loop connecting it to TM8 (loop7, L7b), plus one residue in TM4, mediate tight trimer contacts. Arg299 in H7 forms a salt bridge to Asp288 in the neighbouring protomer. A hydrogen bond connects the hydroxyl of Thr304 in L7b to the backbone carbonyl oxygen of Asn284 the adjacent protomer, and a water molecule connects Asp305 to the backbone amide nitrogen of Gly308 in L7b to Glu132 in TM4. The strong, polar or ionic contacts in the CaiT trimer are thus very different from the hydrophobic trimer contacts in the BetP⁶, even though the order and arrangement of helices within the protomers is the same.

1.2 Inverted repeats in CaiT

TM3 to TM7 and TM8 to TM12 in CaiT define the two halves of an inverted repeat (figs. S2A, S4), as in many other secondary transporters⁶⁻¹². The inner core of the protomer is formed by TM3, TM4 of the first repeat and TM8, TM9 of the second repeat, arranged as an antiparallel four-helix bundle (figs. S2A, S4). This inner core is separated by a cytoplasmic

(L4, IH4) and a periplasmic helix-loop-helix motif (EH9a, L9, EH9b) from the supporting framework comprising TM5 to TM7 and TM10 to TM12. TM1 and the curved TM2 form a clamp-like scaffold for the helices of the inverted repeats (fig. S4).

1.3 Role of methionine in substrate coordination

Although the methionine side chain is usually thought of as hydrophobic, it can in fact participate in polar interactions, because the large, uncharged sulfur is more easily polarized than smaller atoms. Two types of interactions are possible¹³. The methionine sulfur is either negatively polarized and behaves as a nucleophile towards positively charged binding partners (e.g. Na^+ , Ni^{2+} , $\text{H}^{\delta+}$, $\text{C}^{\delta+}$). Alternatively, the methionine sulfur can be positively polarized and behave as an electrophile towards anions or atoms with a partial negative charge (e.g. Cl^- , $\text{O}^{\delta-}$). In CaiT the sulfur of Met331 interacts with the negatively charged carboxyl group of γ -butyrobetaine, providing an elegant solution to the problem of recognizing and coordinating a hydrophilic substrate in the hydrophobic protein interior. It is known from the structures of small organic compounds such as 3-(methylthio) propanoic acid or norbornane endo-acid¹⁴ that sulfur atoms in a covalent bond interact with carboxylates¹⁴⁻¹⁶. Although this interaction does occur in other proteins¹⁷, a functionally important role of such a methionine-carboxylate bridge has not been reported up to now.

2 Tables

Table S1

Structure comparisons of PmCaiT and EcCaiT to each other and to the structurally related transporters BetP, vSGLT and LeuT.

| Mol A | Mol B | Z-Score | Aligned Residues | RMSD (Å) | Sequence Identity (%) |
|--------------------------|--------------------------|----------------|-------------------------|-----------------|------------------------------|
| PmCaiT (Trimer) | EcCaiT (Trimer) | 53.6 | 972 | 0.9 | 87 |
| PmCaiT (Protomer) | EcCaiT (Protomer) | 60.5 | 496 | 0.7 | 87 |
| | | | | | |
| PmCaiT (Trimer) | BetP (Trimer) | 43.1 | 1348 | 2.2 | 25 |
| PmCaiT (Monomer) | BetP (Monomer) | 43.7 | 478 | 2.2 | 25 |
| | | | | | |
| EcCaiT (Trimer) | BetP (Trimer) | 37.2 | 895 | 2.3 | 25 |
| EcCaiT (Monomer) | BetP (Monomer) | 44.0 | 481 | 2.2 | 25 |
| | | | | | |
| PmCaiT (Monomer) | vSGLT (Monomer) | 13.7 | 334 | 4.2 | 13 |
| EcCaiT (Monomer) | vSGLT (Monomer) | 13.6 | 333 | 4.0 | 12 |
| | | | | | |
| PmCaiT (Monomer) | LeuT (Monomer) | 14.8 | 342 | 4.1 | 10 |
| EcCaiT (Monomer) | LeuT (Monomer) | 14.7 | 335 | 4.3 | 10 |

Table S2

Lookup table for trans-membrane helices in LeuT-type transporters. TM3 to TM7 in CaiT or BetP are the equivalent of helices 1 to 5, and TM8 to TM12 are the equivalent of helices 6 to 10 in the other transporters.

| | CaiT | BetP | vSGLT | LeuT | Mhp1 | AdiC | ApcT |
|-----------------|-------------|-------------|--------------|-------------|-------------|-------------|-------------|
| Repeat 1 | | | | | | | |
| Helix 1 | 87 – 118 | 137 – 169 | 52 – 80 | 10 – 38 | 28 – 55 | 11 – 37 | 9 – 37 |
| Helix 2 | 127 – 163 | 177 – 212 | 83 – 109 | 40 – 72 | 57 – 86 | 43 – 67 | 40 – 66 |
| Helix 3 | 186 – 224 | 234 – 268 | 123 – 158 | 87 – 125 | 99 – 137 | 81 – 112 | 83 – 117 |
| Helix 4 | 228 – 249 | 275 – 296 | 161 – 178 | 165 – 185 | 142 – 159 | 122 – 143 | 122 – 141 |
| Helix 5 | 251 – 277 | 300 – 325 | 185 – 213 | 189 – 214 | 161 – 191 | 146 – 172 | 145 – 172 |
| Repeat 2 | | | | | | | |
| Helix 6 | 311 – 340 | 358 – 390 | 249 – 277 | 240 – 269 | 208 – 234 | 195 – 217 | 183 – 214 |
| Helix 7 | 343 – 377 | 393 – 427 | 279 – 314 | 275 – 306 | 241 – 278 | 226 – 248 | 218 – 247 |
| Helix 8 | 403 – 435 | 448 – 482 | 348 – 385 | 336 – 371 | 295 – 331 | 277 – 310 | 269 – 305 |
| Helix 9 | 445 – 467 | 488 – 511 | 391 – 418 | 374 – 396 | 335 – 351 | 323 – 342 | 320 – 337 |
| Helix 10 | 469 – 502 | 513 – 546 | 422 – 448 | 398 – 425 | 359 – 383 | 351 – 376 | 339 – 364 |

Table S3

Kinetic and substrate binding analysis of wildtype (wt) PmCaiT, PmCaiT mutants, and wt EcCaiT.

| | PmCaiT | | | | EcCaiT |
|---|------------------------------------|----------|------------------|------------------------------------|---------------------------------------|
| | wt | E111A | W316A | M331V | wt |
| Transport | | | | | |
| K_M (μ M) | 46 ± 6 119.7 ± 20^a | inactive | 159.2 ± 10.8 | 123.3 ± 12.2 | 81.1 ± 11.8 100.4 ± 14.1^a |
| v_{max} (nmol substrate/ (min•mg protein)) | 4672 ± 205 4824 ± 301^a | | 1816 ± 353 | 478 ± 53 | 4921 ± 243 4975 ± 243^a |
| k_{cat} (L-carnitine/min) | 263 ± 12 272 ± 17^a | | 69 ± 4 | 26 ± 3 | 279 ± 14 282 ± 14^a |
| Substrate binding (detergent solution) | | | | | |
| K_D (γ -butyrobetaine) (mM) | 3.1 ± 0.6 3.4 ± 0.2^a | | 17.5 ± 6.0 | 11.7 ± 1.2 11.8 ± 1.7^a | 3.9 ± 0.7 |
| B_{max} (γ -butyrobetaine) (mM) | 42.6 ± 2.4 42.0 ± 1.0^a | | 58.6 ± 7.8 | 51.1 ± 3.0 50.2 ± 2.8^a | 43.3 ± 3.2 |
| Hill coefficient | 1.0 ± 0.1 | | 0.9 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.1 |
| K_D (L-carnitine) (mM) | 1.8 ± 0.3 2.4 ± 0.4^a | | 9.0 ± 1.3 | 7.1 ± 1.1 7.0 ± 0.8^a | 1.9 ± 0.2 |
| B_{max} (L-carnitine) (mM) | 40.3 ± 2.5 39.1 ± 2.3^a | | 33.7 ± 1.3 | 41.7 ± 2.7 45.1 ± 1.5^a | 39.1 ± 3.1 |
| Hill coefficient | 1.1 ± 0.1 | | 1.0 ± 0.1 | 1.1 ± 0.1 | 1.2 ± 0.1 |
| Substrate binding (proteoliposomes) | | | | | |
| K_D (γ -butyrobetaine) (mM) | 5.6 ± 0.8 | | 11.2 ± 1.7 | n.d. ^{b)} | 5.3 ± 0.8 |
| B_{max} (γ -butyrobetaine) (mM) | 34.3 ± 2.9 | | 21.8 ± 1.8 | n.d. | 22.8 ± 0.8 |
| Hill coefficient | 1.5 ± 0.1 | | 1.7 ± 0.2 | n.d. | 1.4 ± 0.1 |
| K_D (L-carnitine) (mM) | 4.5 ± 0.8 | | 7.5 ± 1.2 | n.d. | 5.8 ± 1.0 |
| B_{max} (L-carnitine) (mM) | 28.2 ± 1.9 | | 24.4 ± 0.8 | n.d. | 30.5 ± 1.5 |
| Hill coefficient | 1.4 ± 0.1 | | 1.6 ± 0.1 | n.d. | 1.5 ± 0.2 |

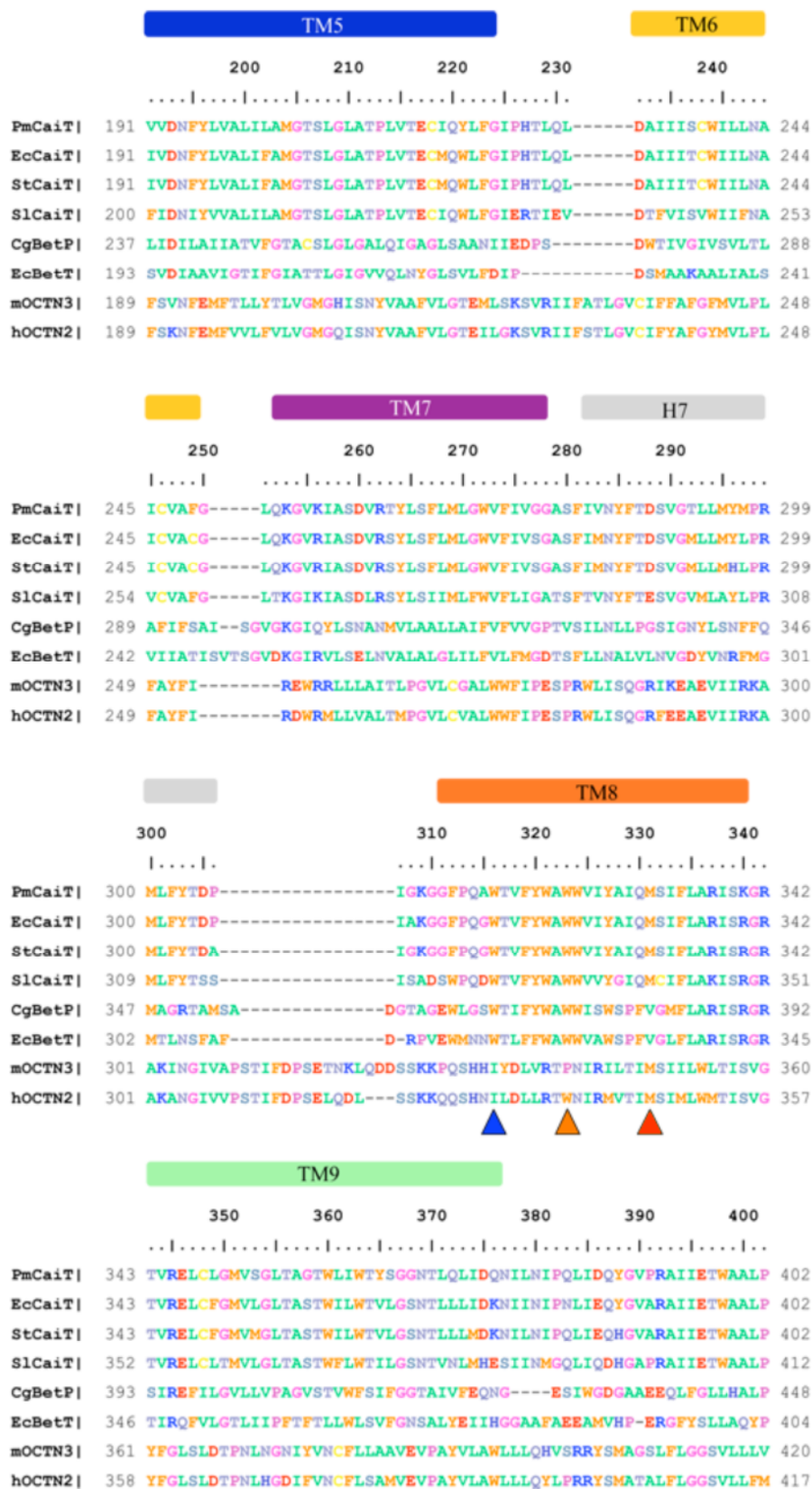
^{a)} Measurements in presence of 50 mM NaCl; ^{b)} not determined

Table S4

Data collection and phasing statistics

| | <i>P. mirabilis</i> CaiT | <i>E. coli</i> CaiT |
|--------------------------------------|---|---|
| Beamline | X10SA SLS | X10SA SLS |
| Wavelength (Å) | 0.92 | 0.98 |
| Resolution (Å) ^{a)} | 19.7 – 2.3 (2.4 – 2.3) | 24.7 – 3.4 (3.6 – 3.4) |
| Cell dimensions | $a = b = 129.2 \text{ Å}, c = 160.3 \text{ Å}$ $\alpha = \beta = 90^\circ, \gamma = 120^\circ$ | $a = b = 124.20 \text{ Å}, c = 154.63 \text{ Å}$ $\alpha = \beta = 90^\circ, \gamma = 120^\circ$ |
| Number of measured reflections | 246 131 | 136 423 |
| Number of unique reflections | 43 243 | 71 232 |
| Completeness (%) | 97.7 (91.2) | 97.7 (87.1) |
| Redundancy | 5.7 | 1.9 |
| $I/\sigma(I)$ | 9.0 (1.4) | 12.8 (1.3) |
| R_{merge} (%) ^{b)} | 8.4 (55.7) | 6.0 (90.0) |
| R_{work} (%) ^{c)} | 20.9 | 23.7 |
| R_{free} (%) ^{d)} | 23.8 | 27.1 |
| r.m.s. deviations | | |
| Bond length (Å) | 0.007 | 0.011 |
| Bond angles (°) | 0.980 | 1.302 |

^{a)} Numbers in parentheses represent statistics for data in the highest resolution shell^{b)} $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i ||F_{\text{hkl}}| - |F_{\text{hkl}}(i)|| / \sum_{\text{hkl}} \sum_i |F_{\text{hkl}}(i)|$ ^{c)} $R_{\text{work}} = \sum_{\text{hkl}} ||F_{\text{obs}}| - k|F_{\text{calc}}|| / \sum_{\text{hkl}} |F_{\text{obs}}|$ ^{d)} R_{free} was calculated with 5% of reflections not used during refinement.



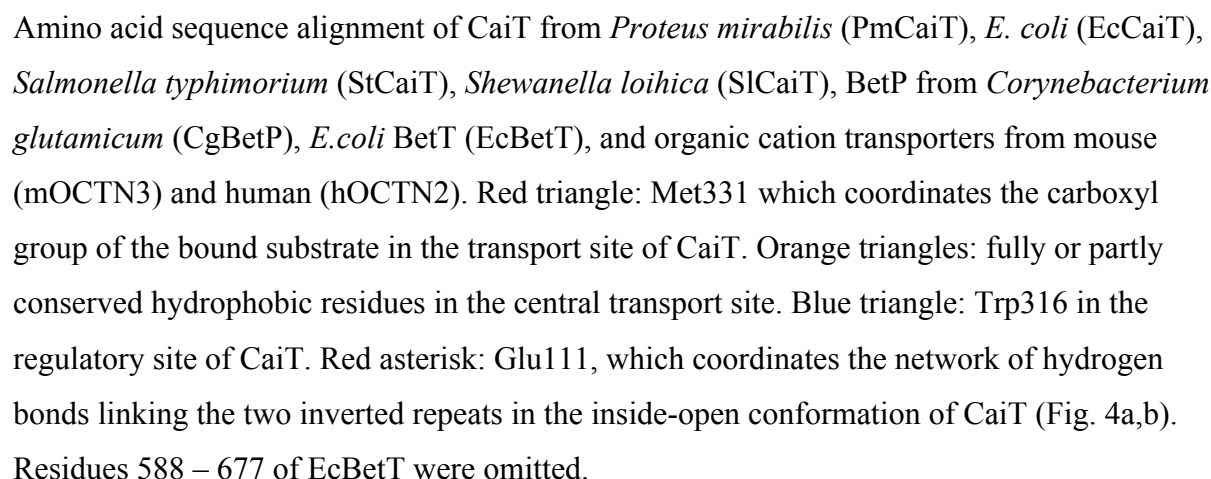
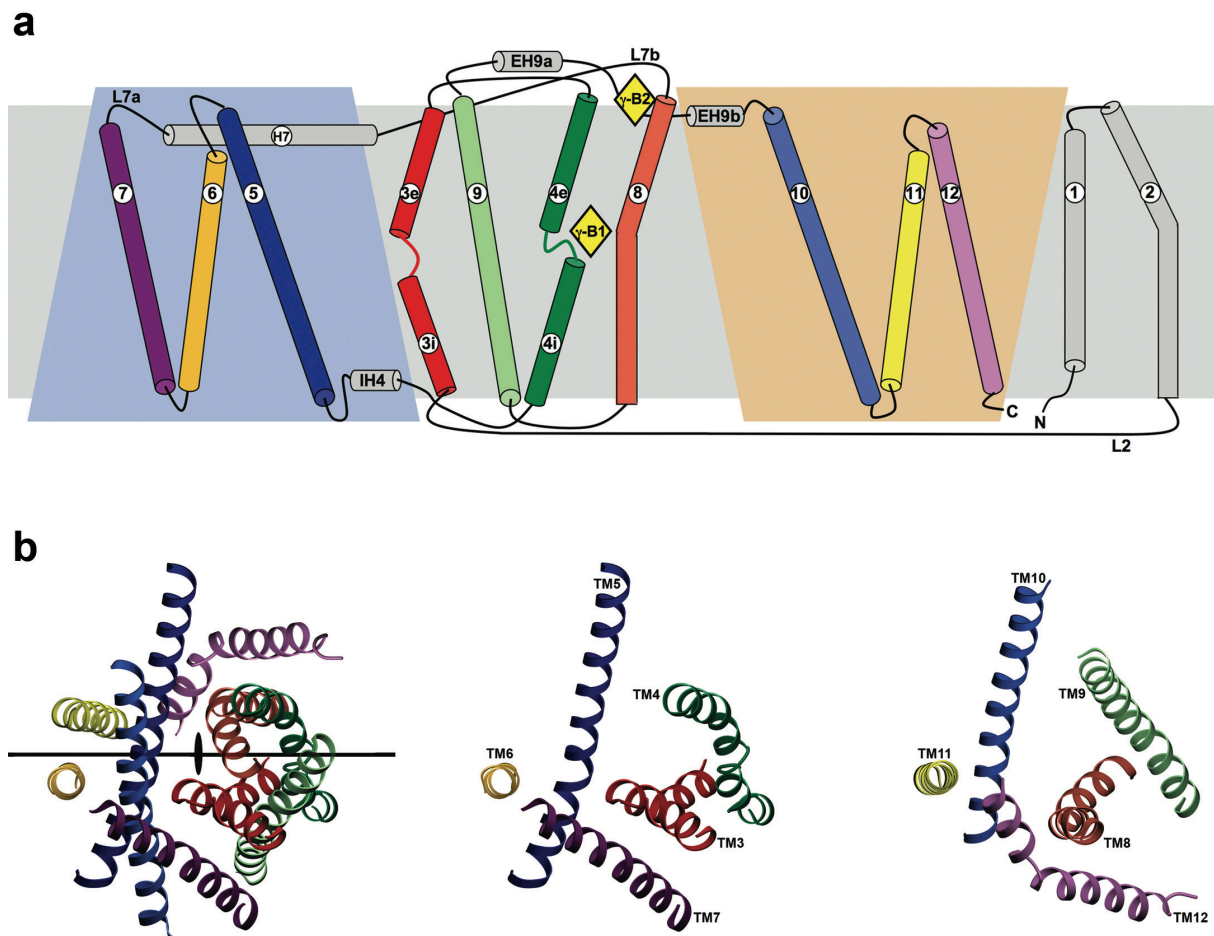


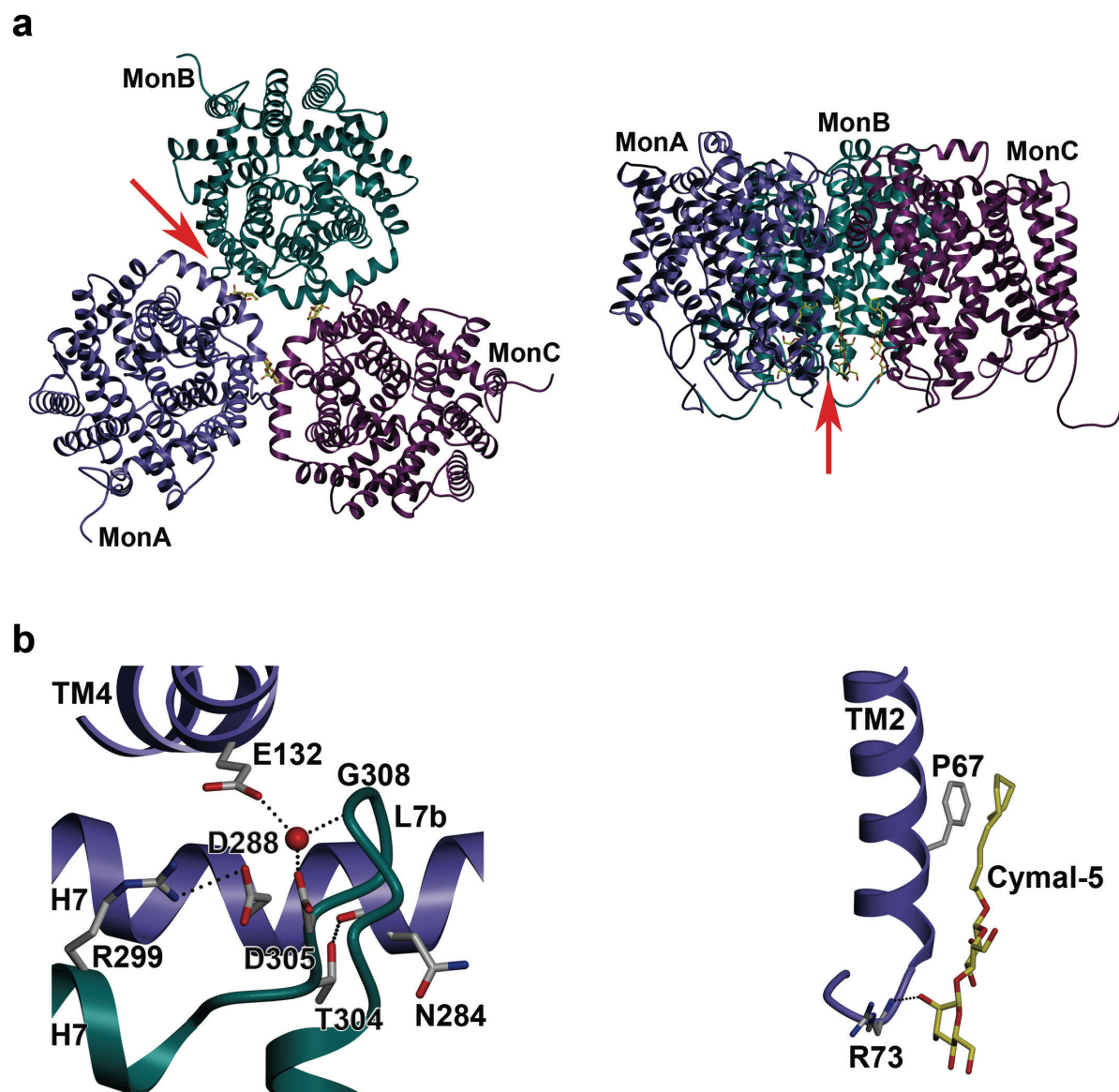
Figure S 2



(a) Inverted helix repeats in CaiT. Repeat 1 (TM3 to TM7) is shown in dark colours, repeat 2 (TM8 to TM12) in lighter colours.

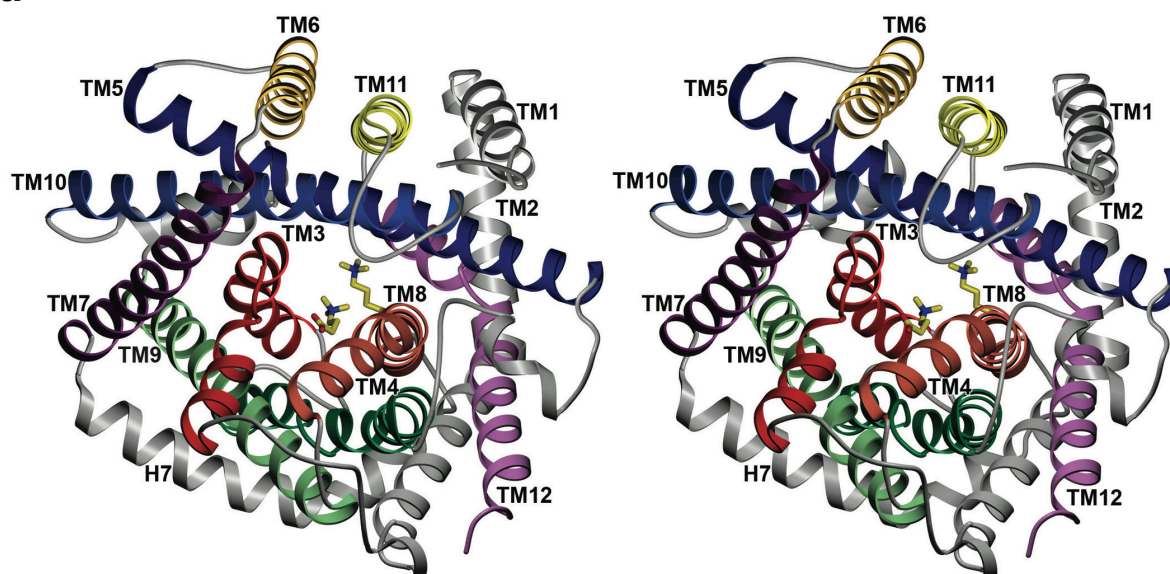
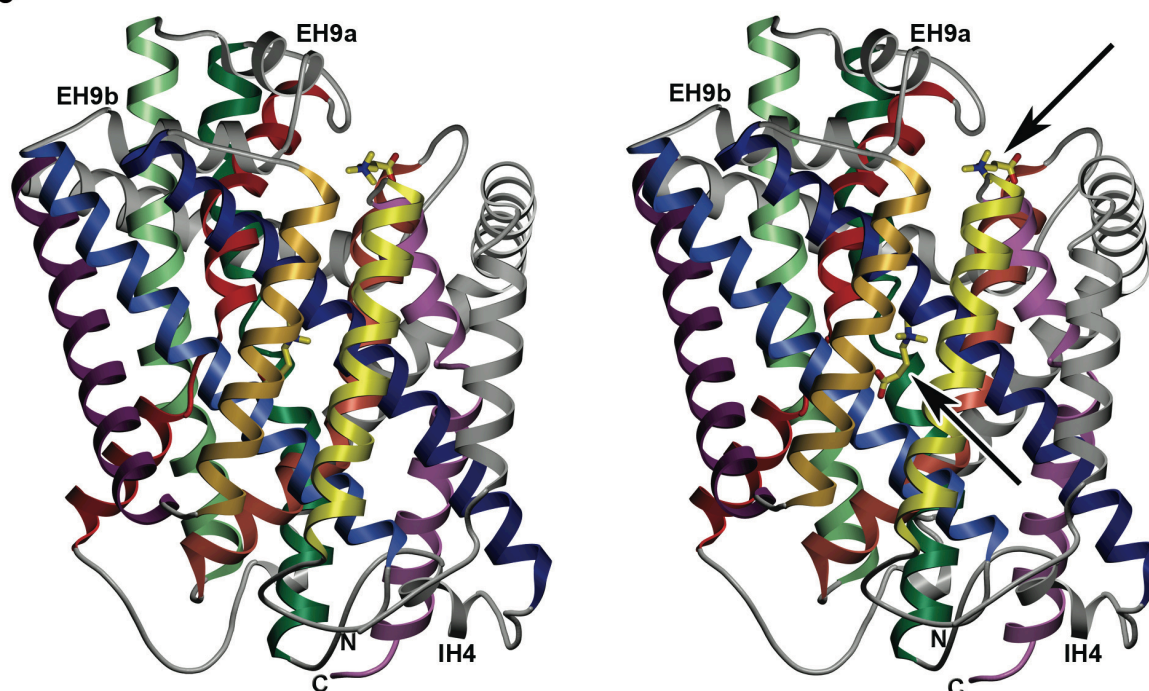
(b) The 10 TM helices of the two inverted repeats (left) are coloured as in (a). Repeat 2 (right) is rotated relative to repeat 1 (centre) by $\sim 180^\circ$ around the internal pseudo-twofold axis of the protomer (black line), and by $\sim 20^\circ$ in the perpendicular direction.

Figure S 3



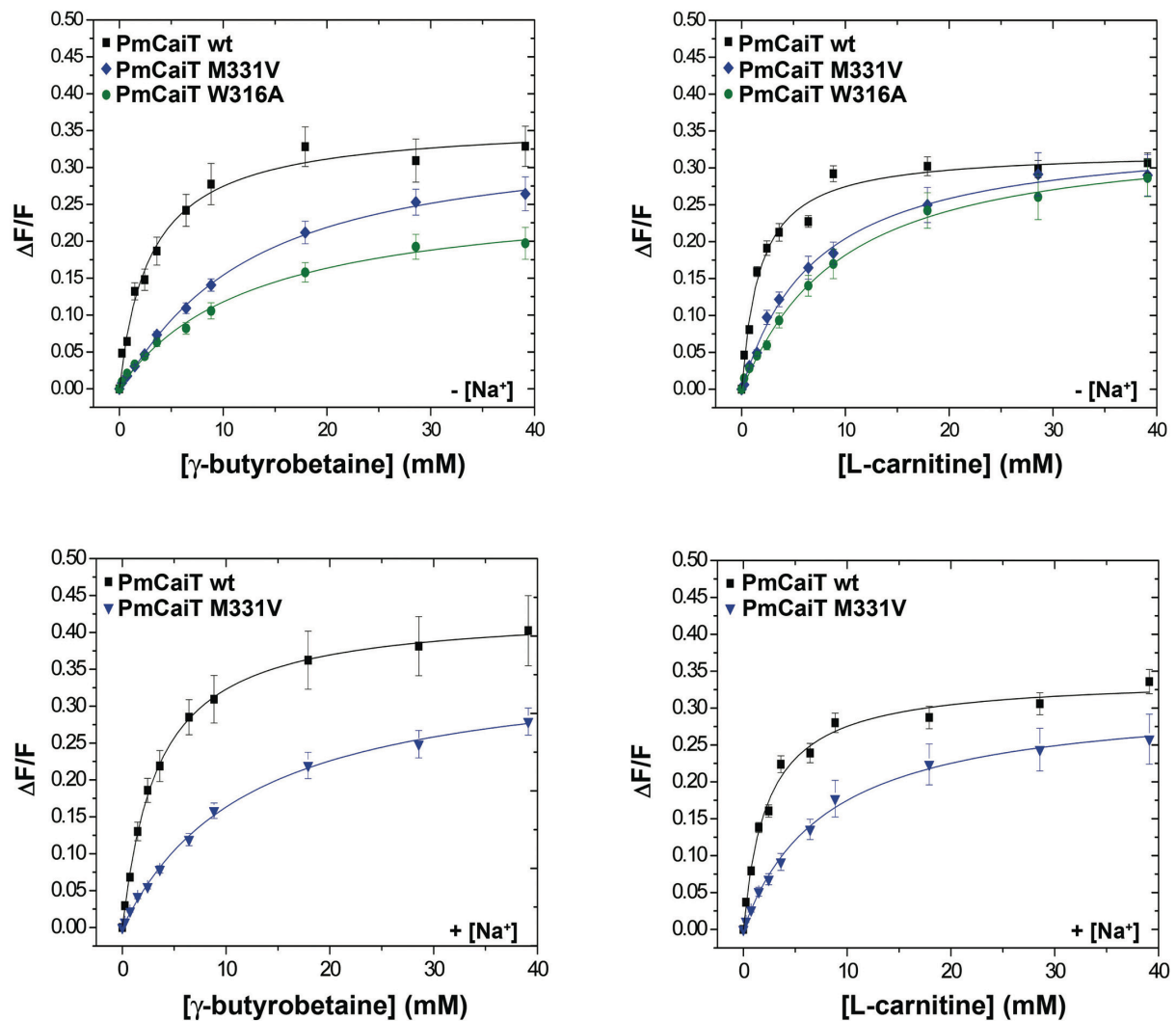
(a) The PmCaiT trimer. In the periplasmic view (left), close contacts between protomers (arrow) are mediated by an ion pair (yellow sidechains) and hydrogen bonds. The side view (right) shows a deep cavity between protomers on the cytoplasmic side (arrow) that contains bound detergent (yellow). (b) Detailed views of the ion and water bridges that connect protomers in the trimer (left), and the Cymal-5 detergent head group interacting with the aromatic sidechain of Phe67 (right) in the hydrophobic cavity.

Figure S 4

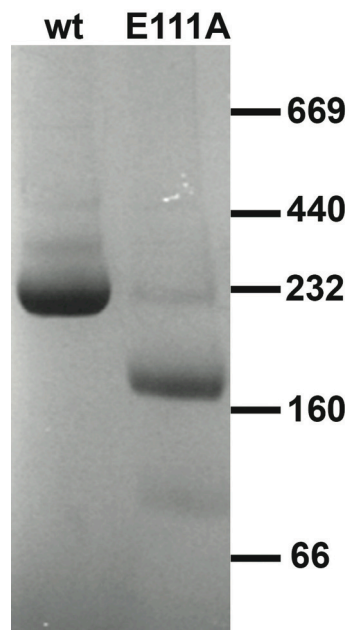
a**b**

Stereo diagrams of the EcCaiT protomer with two bound γ -butyrobetaine substrates. (a) cytoplasmic view; (b) side view. The arrows in B point to the bound substrate molecules in the transport site (centre) and in the regulatory site on the periplasmic side.

Figure S 5

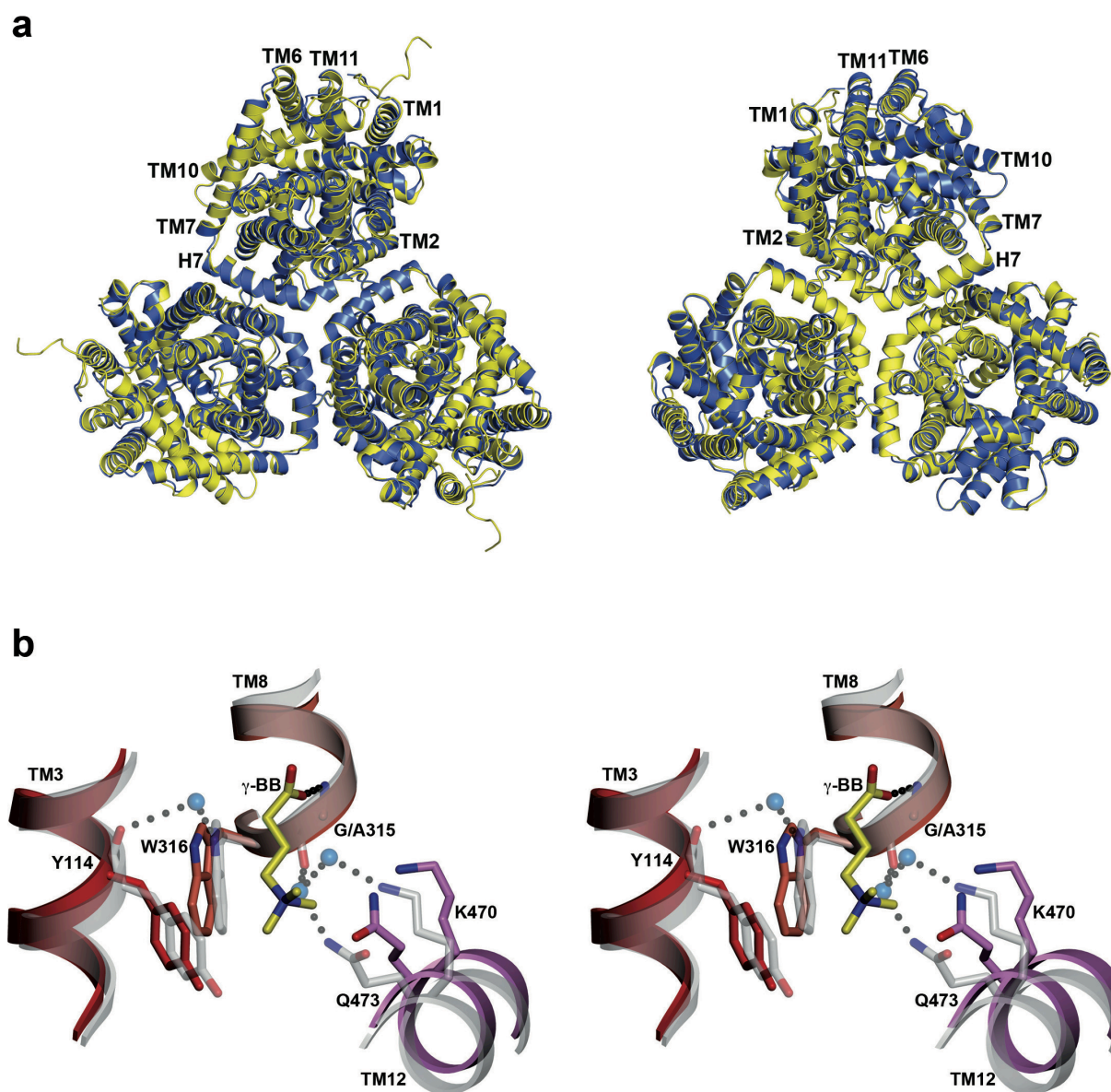


Binding of γ -butyrobetaine or L-carnitine to wildtype (wt) PmCaiT (black), PmCaiT M331V (blue) and PmCaiT W316A (green) solubilised in the detergent Cymal-5 in sodium-free buffer (top) or with 50 mM NaCl, monitored by Trp fluorescence.

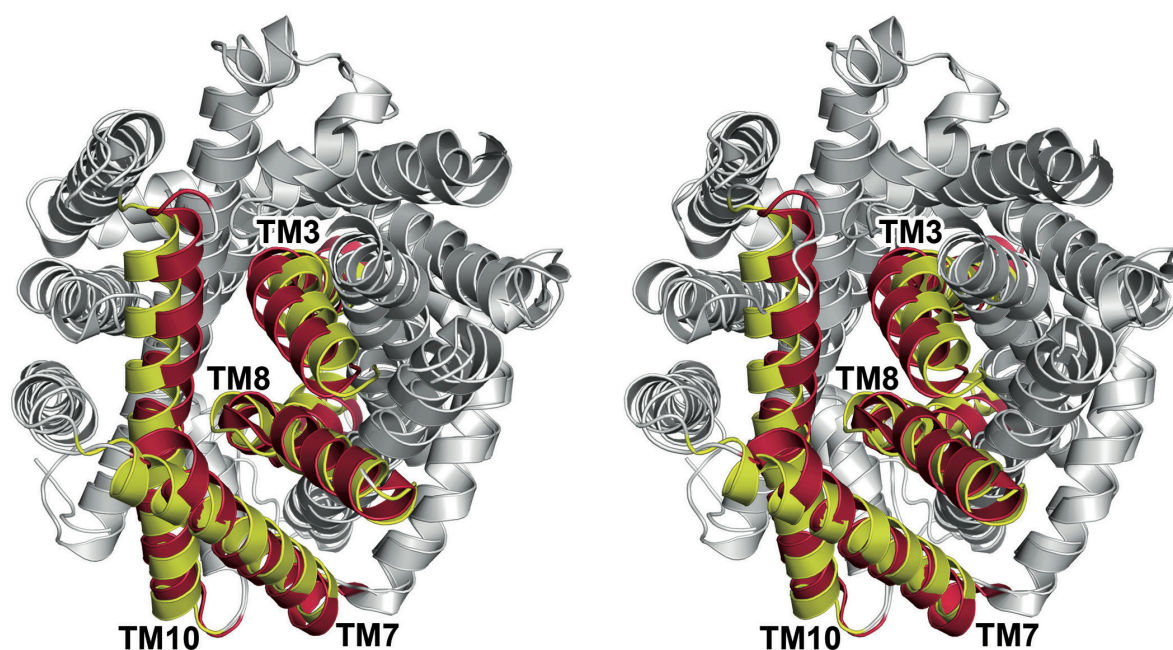
Figure S 6

Oligomeric state of CaiT in detergent solution. Wildtype PmCaiT (wt) forms stable trimers that run at about 230 kDa in the blue-native gradient gel (4 – 16%). Mutation of Glu111 to alanine (E111A) destabilizes the trimer, and results in the appearance of dimers and monomers.

Figure S 7

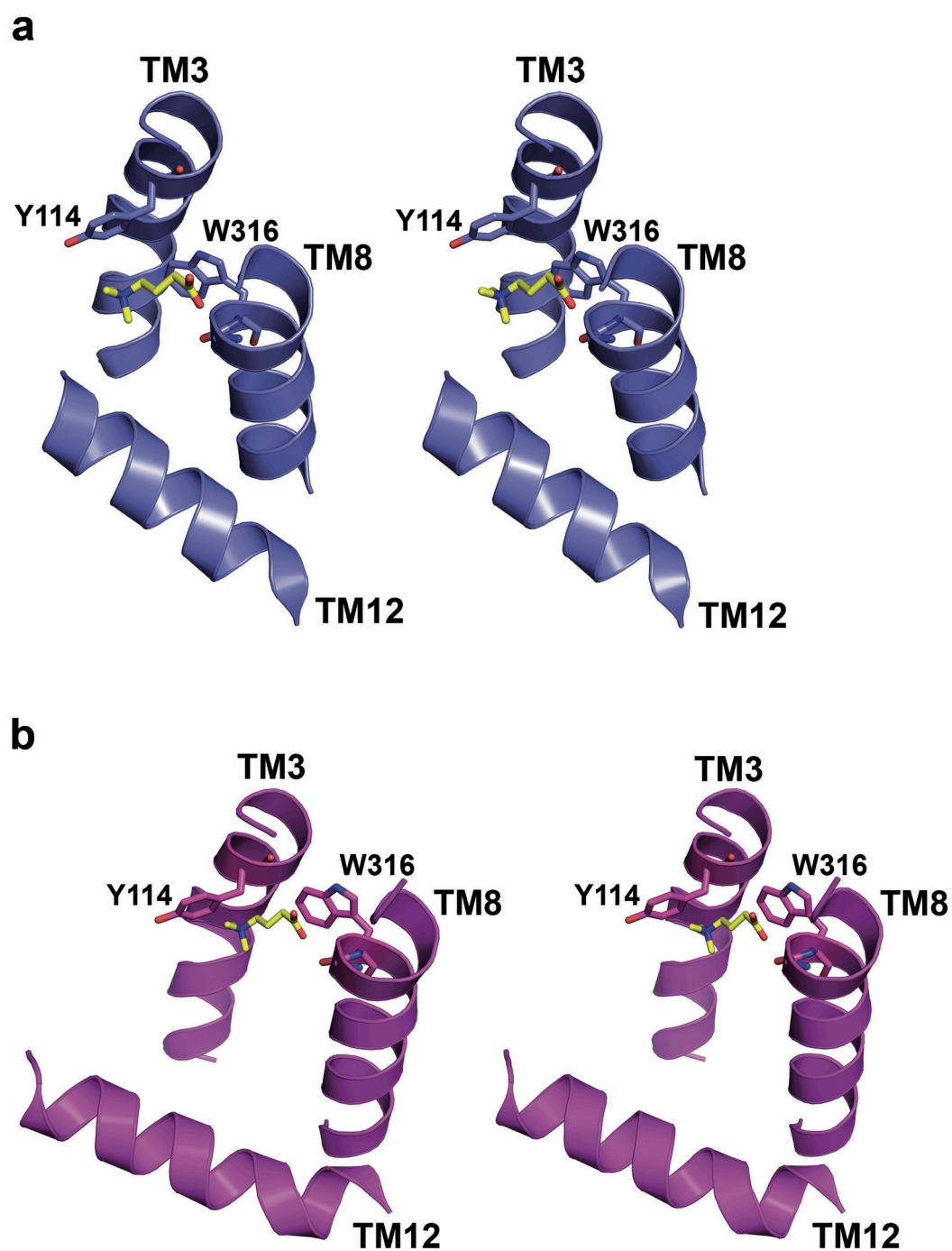


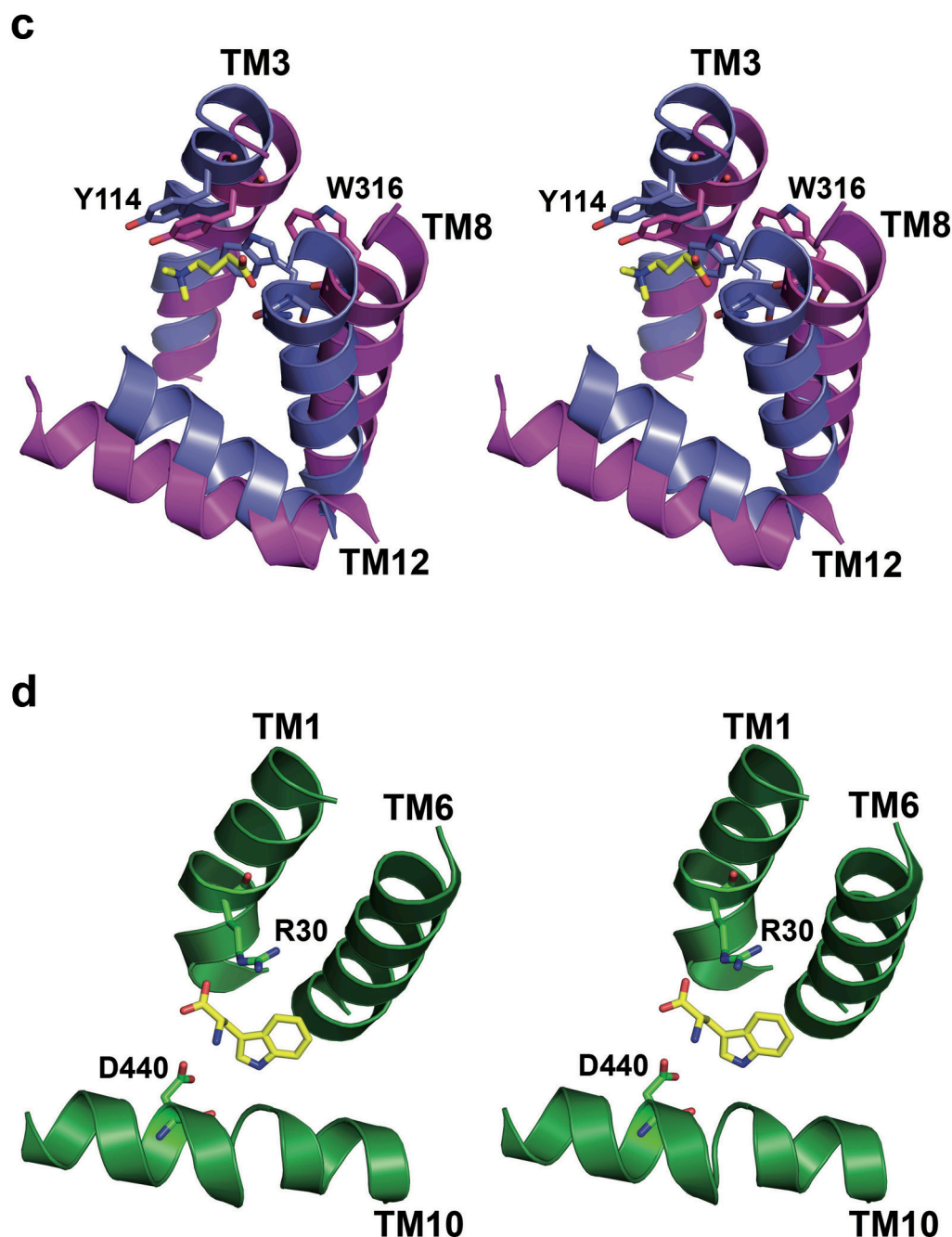
(a) Cytoplasmic (left) and extracellular view (right) of the PmCaiT trimer (yellow) superposed onto the EcCaiT trimer (blue) in stereo. The superposition indicates a 3° tilt of the substrate-bound EcCaiT protomer relative to PmCaiT, towards the threefold axis in the extracellular view. (b) Stereo view of the superimposed regulatory sites of EcCaiT (coloured) and PmCaiT (grey). Substrate binding in the external binding site replaces two water molecules and disrupts the hydrogen bond network coordinated by them. As a result, the extracellular end of TM12 moves by ~1.3Å.

Figure S 8

Stereo drawing of superposed protomers of PmCaiT and BetP, showing an iris-like movement of the cytoplasmic part of the helices TM3, TM7, TM8 and TM10 (yellow for PmCaiT, red for BetP) in the transition from the fully inside-open conformation of CaiT to the occluded state of BetP.

Figure S 9





Stereo drawings comparing EcCaiT helices TM3, TM8 and TM12 with key residues in the inside-open conformation (a, blue) to a model of EcCaiT in the outside-open conformation (b, pink), based on the LeuT structure. In the model (b), the second, regulatory substrate-binding site on the extracellular side defined by residues Tyr114, Trp316 and the carbonyl oxygen of Gly315 remains intact, so that substrate is likely to remain bound throughout the transport cycle. (c) Superposition of A and B, indicating the helix movements that accompany the transition from the inside-open to the outside-open conformation on the extracellular side of CaiT. (d) Corresponding helix regions of the outside-open LeuT (TM1, TM6 and TM10,

green) in complex with tryptophan. The Trp in the extracellular channel of LeuT is in a different position compared to the substrate in the regulatory site of CaiT.

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